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Gonadal Hydration of Carp *(Cyprinus carpio)* and Goldfish *(Carassius auratus)* After Injections of Pituitary Extracts[1]

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(Text-figures 1-15)

INTRODUCTION

THE pituitary of carp contains a principle controlling the preparation of semen for spawning in carp, trout *(Salmo gairdneri)* and a number of other species of fish. Clemens & Grant (1964) demonstrated that changes in the water and salt content and sperm cell densities of semen occur in direct proportion to dosages of water extract from whole ground acetone-dried pituitaries of carp and that these responses, being relatively rapid and sensitive, provide an assay for following the activity in chemical isolation and purification procedures. Although the seminal fluidity response provides advantages, such as an hourly pattern of response changes in the same test animal, it is impractical to use with small fish where the semen cannot be collected in sufficient quantity, and gives no biological comparison in female fish.

Since our main purpose was to pursue the role of gonadotropins in the reproductive cycle of fish, we needed a method of measuring a response to pituitary extracts giving a reliable comparison among fish of different size, maturity and sex. We needed to know further the role of electrolytes and water in the reproductive cycle in nature. The phase reported here reveals that water and electrolyte changes can be induced in the gonads of either sex and that these changes are an essential part of the reproductive cycle, in particular, the spawning and prespawning gonadal processes. Data currently collected, to be presented in an additional paper establishing the existence of a water and electrolyte cycle in fish gonads in nature, along with the experimental data presented in this paper, offer convincing evidence that the gonadal hydration of fish is hormonally controlled and regulated by the pituitary gland.

METHODS

Both species of experimental fish, carp and goldfish, were maintained on a year-around basis in the University of Oklahoma Fisheries Research Center ponds which provided us with fish of known history. Fish were seined from the ponds and acclimated in 100-gallon metal troughs at an experimental temperature of 22 ± 0.5° C for a period of one week to ten days before an experiment. Fish were fed (3% body weight) a commercial fish food (37% protein) five days a week. Feeding was discontinued 24 hours before the experiment. Only mature, healthy specimens were used.

Acetone-dried carp pituitaries were purchased for experimentation from Stoller Fisheries, Iowa. Whole pituitaries were ground, extracted with distilled water and injected intraperitoneally. The injection material was prepared as follows: Male and female carp pituitary glands collected in January and dried with four 12-hour changes of acetone were crushed, extracted with distilled water at room temperature for 30 minutes and filtered. The filtrate solution was then diluted with distilled water to the desired concentrations based on the dry weight of the pituitary glands. Dosages were made in logarithmic proportions and administered on a weight basis, *i.e.*, x mg of acetone-dried, whole pituitaries per 100 gm of fish body weight (excess water blotted from its

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surface). Extract volumes were injected proportional to fish weights, 1.0 cc/100 gm respectively. Gonads were weighed to the nearest gram and "fat-free" pieces (250-650 mg) were placed on tared glass cover slips, macerated and weighed to the nearest 0.2 mg. The weight lost on drying overnight at 65° C was accepted as the water loss.

Deviations from the above practices are mentioned in the text.

Results

Seminal Response

The seminal fluidity of carp (groups of five fish, 450 ± 50 gm) peaked approximately 24 hours after injection (homoplastic pituitary extract), then gradually subsided until the base level was reached some three to four days later. The curve is skewed to the left, indicating that for *ca.* 24 hours after injection there was a more rapid movement of water into the testes (semen) than out.

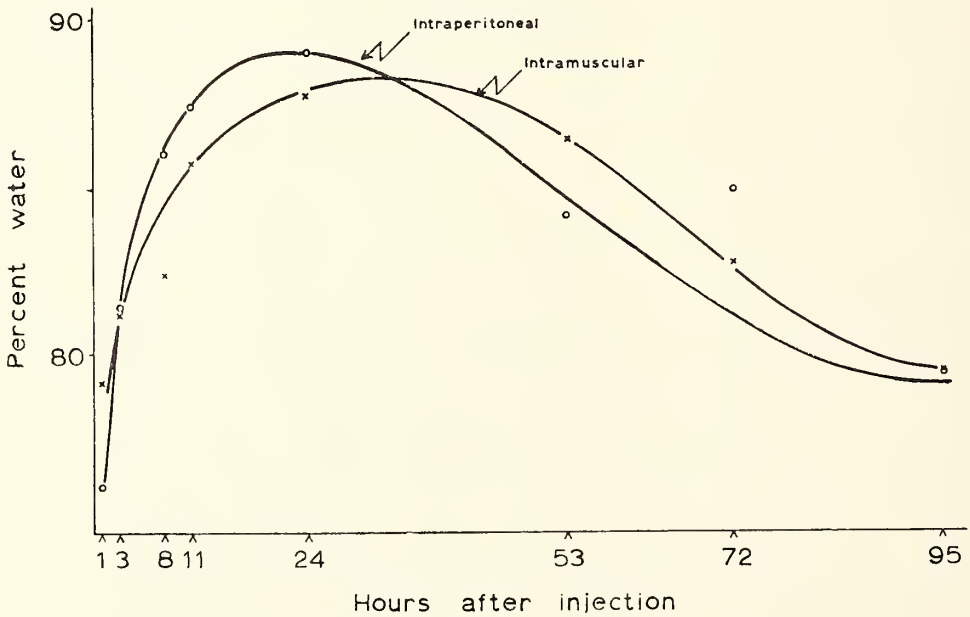
The experiment exemplified by Text-fig. 1 was conducted to determine whether the muscle or the body cavity was the more suitable site for injection. The levels of response in the two types of injections were not significantly different.

Testicular Response

A testis was removed from a mature carp injected 24 hours previously with a water extract from 1.0 mg of whole ground acetone-dried carp pituitaries. Its length was divided into ten parts and the relative water percentage was determined for a fraction of each part. Values for all ten parts of the testes were similar with the exception of sample eight which was apparently a technical error. A gain in water content appeared throughout the entire length of the organ and no part of the testis contained more water than another in gross analysis (Table I). Since the testes appeared uniformly hydrated in our experiments, it remained then for us to establish whether or not testicular hydration could be correlated with hormonal injections and the seminal fluidity response.

The water content of both the semen and the testes of injected fish increased proportionally with the dosage (0.69 to 69 micrograms/gm range with half-log dosage increases) (Text-fig. 2). The relative water content of the testes was lower and results were more uniform between dosages. This demonstrated that the seminal fluidity response was reflected in gonadal hydration and that the testes could be used to measure the hormonally induced response.

Since this experiment, several hundred male goldfish have been used throughout the course of the year in our assay work. The data for these fish were used in determining the standard curve (Text-fig. 3). The assay range is represented by the straight part of the sigmoid curve between 1.0 and 100 μg/gm (0.1 and 10 mg/100 gm in practice). The standard deviation, standard error



Text-fig. 1. The mean (five carp) seminal hydration response with respect to time after a single injection of homoplastic pituitary extract.

Table I. The Water Percentages of Ten Portions of Carp Gonad Equally Spaced Throughout Its Length, with Respect to the Duration of Drying

Part No.	6 hrs.	24 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	Mean[1]	SD
1	80.4	79.5	79.2	79.9	80.8	80.3	79.9	0.64
2	75.7	78.8	78.8	79.3	79.4	79.3	79.1	0.30
3	75.7	79.3	79.4	79.2	80.0	79.6	79.5	0.32
4	77.8	78.6	78.6	79.9	79.9	79.5	79.3	0.66
5	78.7	79.9	79.9	79.5	80.0	79.9	79.8	0.20
6	78.7	79.6	79.3	80.7	80.3	79.6	79.9	0.58
7	77.5	78.2	77.2	77.3	78.5	78.3	77.9	0.61
8	70.2[2]	71.3	71.0	72.6	73.0	72.6	...	...
9	77.9	79.2	78.9	78.7	79.5	79.6	79.2	0.38
10	74.7	78.6	78.6	78.5	79.5	79.0	78.8	0.42
Mean	77.5	79.1	78.9	79.2	79.8	79.5		
SD	1.80	0.87	0.88	0.97	0.65	0.54		

[1]6-hour readings omitted.
[2]Apparently an original error in weighing, calculations omitted.

and the number of fish for the controls and each of the dosages were respectively as follows:

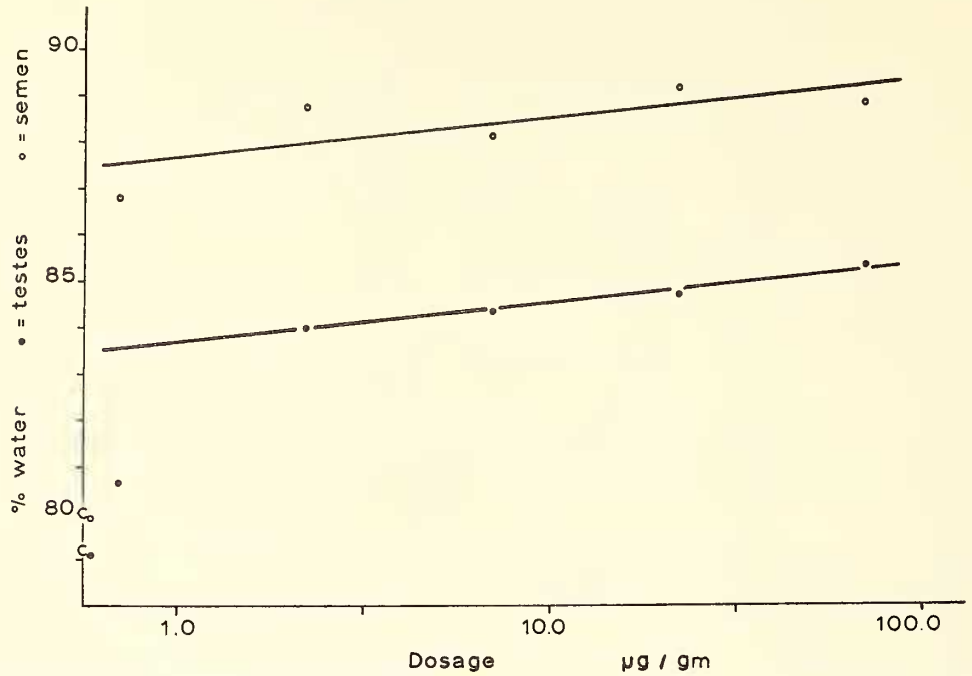
Control	2.46,	.26,	140
0.2 μg/gm	1.79,	.25,	52
0.6 μg/gm	2.09,	.35,	36
2.0 μg/gm	1.76,	.22,	62
6.6 μg/gm	2.24,	.54,	17
10 μg/gm	2.58,	.53,	24
22 μg/gm	2.21,	.27,	68
31.6 μg/gm	1.99,	.48,	17
50 μg/gm	1.47,	.49,	9
100 μg/gm	1.16,	.24,	24
316 μg/gm	.63,	.28,	5

The standard deviations are considerably higher than for each individual experiment since there was a seasonal response variation in the controls and in the experimental fish which approximated the seasonal gonadal water of fish in nature (Text-fig. 4).

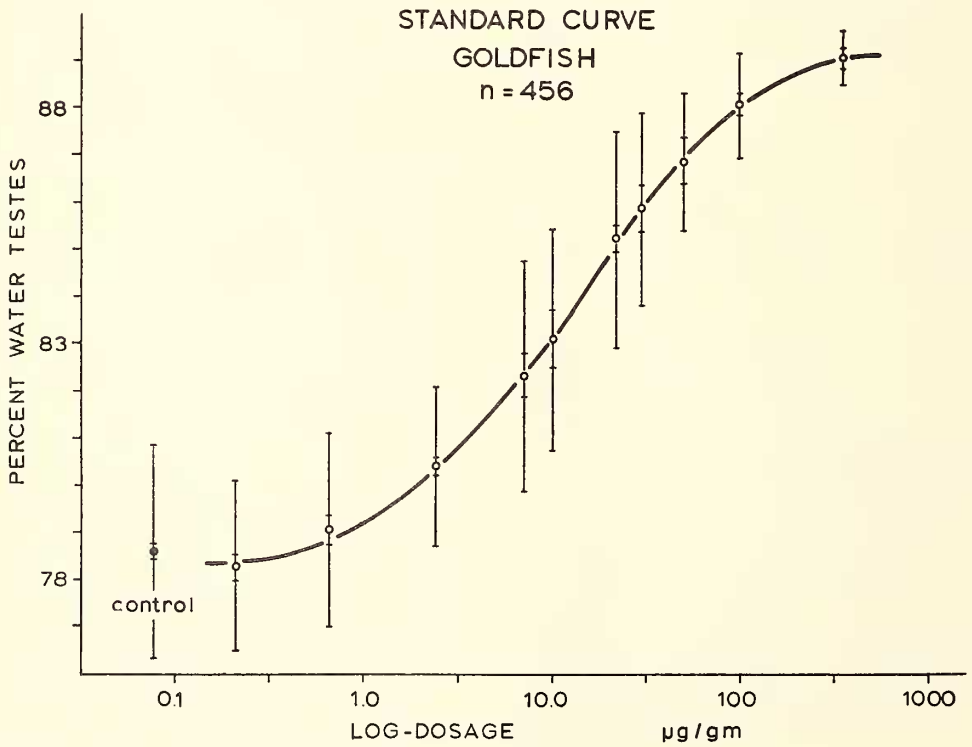
The standard curve represents the entire year. The periods of greatest response appeared during May, June and October. The period in the fall should be considered late September and early October, since the October experiments were conducted during the first ten days of that month and the September ones at the end of the first week of the month. During the spring period of April, May and June, the spawning period, the experimental fish gave the greatest response over that of the controls. For example, the difference between the average monthly response for an injection level of 22 μg/gm and the controls was 6.3 in February, 6.5 in March, 13.0 in April, 11.7 in May, 10.2 in June, 5.3 in July, 5.8 in August, 2.8 in September, 8.6 in October and 8.6 in November. The greatest difference between the maximum and the minimum monthly average during the year was 6.9%; and for the experimental fish receiving 22 μg/gm was 3.3%. The respective standard deviations and errors were 2.5 and .26, and 2.2 and .27. There was greater seasonal variation in our controls than in the injected fish.

The controls showed a significant reduction of gonadal water below the pondfish during the period from April through June, while the two values were approximately the same during the rest of the year. This suggested that some aspect of "handling" or perhaps temperature might be involved. However, the period from October to May when fish were moved from the colder pond water to the warmer experimental water (22° C) included times when the control fish approximated the values of the experimental fish as well as the times when the gonadal water of the control fish was significantly lower than that of the pondfish. The change of temperature from the pond environment to the experimental conditions, therefore, did not seem to be a major factor in explaining the reduction of gonadal water of the controls below the gonadal water of the pondfish.

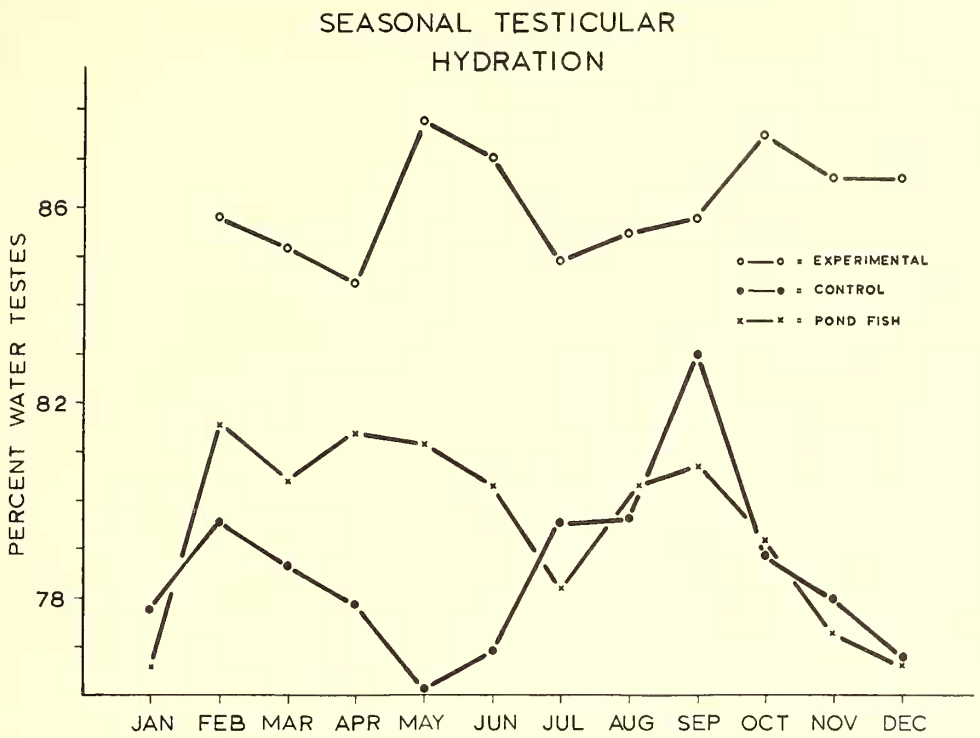
During March, an experiment was conducted to determine the effect of "handling" on the gonadal water of 35 male goldfish. The effects of bringing goldfish from the pond into the laboratory and raising water temperature alone caused a slight decrease which was not statistically significant, while the effects of bringing the fish into the laboratory and raising the water temperature along with the handling involved in their weighing, sexing, fin-clipping and needle puncture caused a 1.6% rise in the gonadal water content ($p=.05$). This was the only step in the handling



TEXT-FIG. 2. A comparison of seminal and testicular hydration, 24 hours after injections of various dosages of homoplastic pituitary extract.



TEXT-FIG. 3. Standard gonadal hydration response curve of male goldfish to injections of carp pituitary extract. Standard deviations and errors are given.



TEXT-FIG. 4. Seasonal gonadal hydration of male goldfish in nature and in injected and non-injected experimental fish.

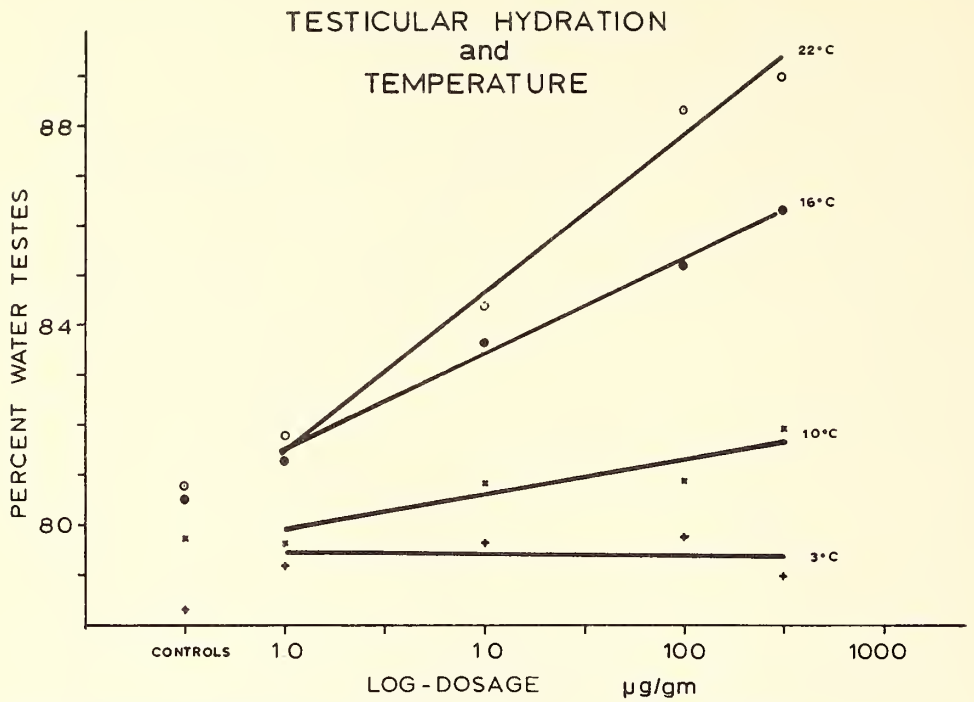
procedure that produced a significant change in the gonadal water content, but this left unexplained the question of why the controls during the spring months were lower than the pondfish.

Another experiment was conducted on 90 male goldfish at the end of April to determine the effects of acclimation under laboratory conditions. The gonadal water of fish removed from pond water at a temperature of 13° C and acclimated at 22° C under laboratory conditions without food declined from 81.9% to 80.6% in five days, to 78.5% in seven days, to 75.0% in 14 days and to 72.9% in 23 days. When food was given, the gonadal water did not change for a test period of the first five days in the laboratory. Fish kept for seven days without food took approximately 16 days on a *daily* diet of 3% per body weight of Clark's trout food (37% protein) to recover the original gonadal water content. These experiments indicated that food was more critical than temperature in our method of handling.

The effect of food on the gonadal hydration re-opened the question of whether or not our laboratory fish were properly fed. Since feeding procedures were the same throughout the year, the most logical explanation lies in the fact that fish during the spawning period require more food than during other times of the year. In another experiment in our laboratory, goldfish that had shown weekly body gains for seven months on a 3% diet (fed five times a week)[2] at a constant temperature (22 ± .5° C) lost weight during the period from the middle of February to the middle of March. The condition was corrected by raising the food to 5% of the body weight[2]. It is unfortunate to have to report that the control fish which were being fed five times a week on a 3% diet were not changed to a 5% diet at this time.

Male goldfish were collected in the pond on March 1, when the temperature was 3.0° C. Groups of 15 were placed in each of the following temperatures: 3.0, 10.0, 16.0 and 22.0° C (± 0.5) and allowed to acclimate for four days before being injected with water extracts of carp pituitaries in dosages of 316, 100, 10, 1.0 μg/gm and controls. The relative water percentage of the testes was measured 24 hours after injection (Text-fig. 5).

[2]Fish fed five times a week on a 3% diet average 2.1% per day per week and the 5% diet reference is also on a five day basis, which then reduces the diet to 3.6% per day per week.



TEXT-FIG. 5. Gonadal hydration response (24 hours) of male goldfish at different temperatures; injected with carp pituitary extract.

The most apparent observation in Text-fig. 5 is that temperature changes the slope of the curve, which was unlike some of the other varied parameters where the slope remained the same but the level of the curve was uniformly depressed. As was expected, as the temperature increased the response increased and the larger dosages became more effective. Although the control fish had the lowest gonadal water at the coldest temperature, it was not statistically significant. This may mean that the endogenous blood level of the hormone in question was low and that at low blood levels temperature was not a significant factor. However, many other possibilities exist, particularly in the area of inhibition. No response was seen for any dosage at 3.0° C, a weak response ($.20 > p > .10$) at 10° C, while marked responses were observed at 16.0 and 22.0° C. Generally speaking, the response for a six-degree change in temperature between 10 and 16° C was about twice the response for a six-degree change of temperature above and below these values. From a practical standpoint dosages were more critical at the higher temperatures. This observation was in keeping with the fact that goldfish spawn in the spring during the time of the year when the temperature is increasing. Thus, if the stimulus for hormonal secretion were present to provide relatively high blood levels, it would not be effective at low temperatures and, under these circumstances, raising the water temperature would be sufficient to induce spawning.

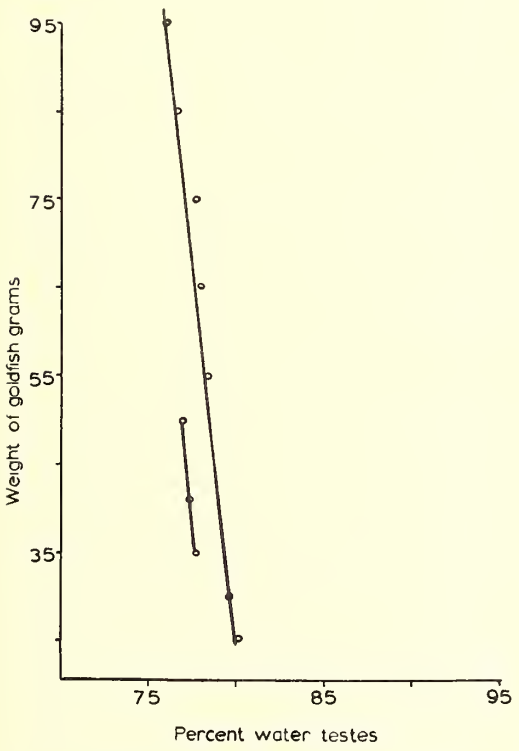
Some assessment of the effect of various factors on induced and non-induced gonadal hydration, such as environmental variations in illumination and dissolved oxygen, as well as variations in types of injections, gonadal fat and forced exercise (physical stress?), was made from the results of pilot studies and more extensive experiments in certain cases.

Male goldfish exhibited a lower gonadal content when kept in the laboratory under continuous light (71.2 and 73.4%) than in total darkness (75.05 and 75.7%) regardless of whether they were maintained in cold (5-7° C) or warm (22° C) water. The testicular water of those fish kept in the dark was not significantly different from pond fish at the end of the experimental period. Continuous light (200-watt bulb about 30 cm above water 25 cm deep) may have kept the fish active throughout the exposure, thereby increasing their food requirements. We think the decreased testicular water of the light-controlled fish resulted from reasons similar to those found in the fish acclimated without food (p. 197).

Circumstantial evidence indicates that in con-

ditions of insufficient dissolved oxygen the response is lowered. Sixty male goldfish, ranging from 22 to 102 gm, were each injected with the same dosage (0.5 mg per fish) and the testicular water percentages were determined 24 hours later. The data plotted in Text-fig. 6 form two parallel lines. It is postulated that the smaller curve, representing 26 fish, resulted from conditions of low oxygen, since these fish occupied the three compartments towards the discharge end of an overloaded tank through which water was circulated.

Fat deposited around the gonad substantially reduced the water content, and therefore was carefully dissected away from any portion of the gonad used in water content determinations. As a precaution, any fish whose gonadal portion appeared oily after oven-drying was excluded from the determination, since such specimens usually had water contents as much as 3% lower than the mean. On the other hand, we did not use fish that were thin and obviously in poor health, since it was an unacceptable practice to use such individuals even though their gonadal water was only about 1% higher than the mean. However, their muscular water was as high as 7% above the mean.



TEXT-FIG. 6. Gonadal hydration response of varying of male goldfish injected with the same dosage of carp pituitary extract. The small curve is believed to show the effect of low environmental oxygen.

This information should serve to emphasize the importance of using healthy fish of known history from the same population in assay work. In the event that different populations are used, the reference curve should be re-standardized.

The effects of non-hormonal substances injected into the body cavity were to lower the response if an effect was observed. For instance, phosphoric acid buffered slightly akaline lowered the gonadal water content of the controls from 82.7 to 81.0% ($p = .005$). However, in another experiment the experimental fish with 79.5% gonadal water were not statistically different from the controls with 80.0% gonadal water. In another experiment the non-buffered phosphoric acid used to carry P^{32} did not alter the gonadal water (74.3 and 74.2%). Perhaps this means that when the endogenous values are high, it is easier to lower the testicular water content by stress than when the hormonal levels are relatively low.

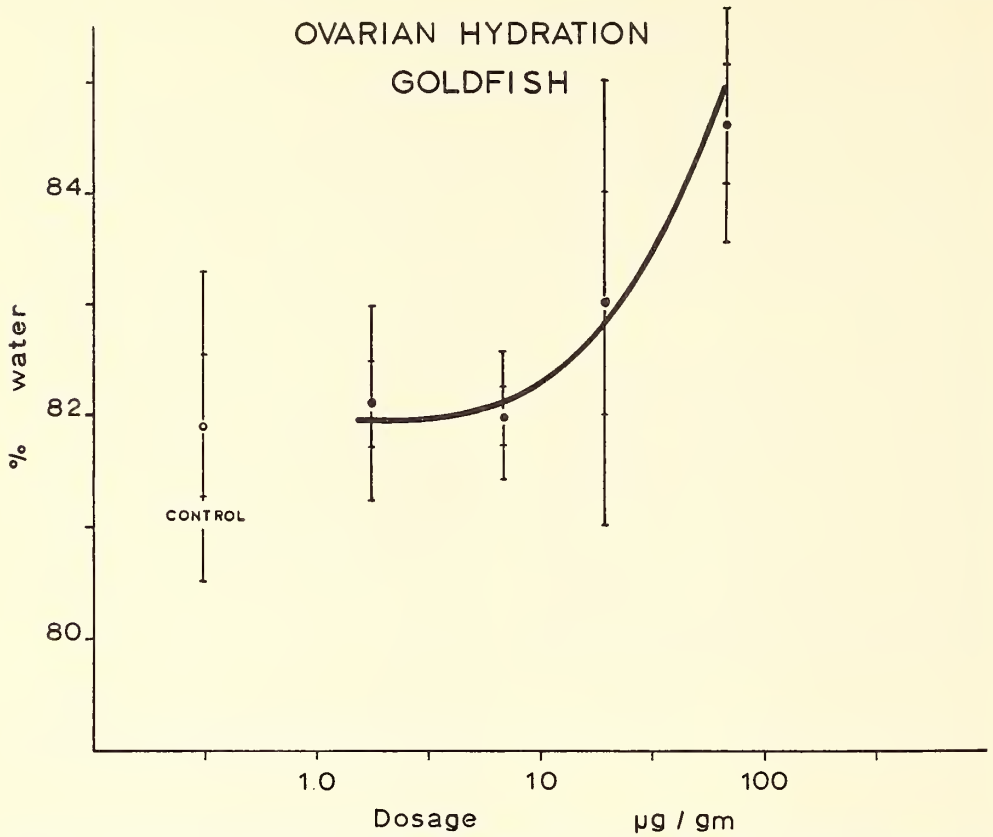
The distilled water used to extract the pituitary activity and to serve as an injection carrier is not in keeping with the osmotically balanced physiological saline normally used for injections. This practice began several years ago (Clemens & Sneed, 1962). No detectable change in gonadal hydration was elicited from a single intraperitoneal injection of distilled water in the amounts we used (up to 1.0 cc/100 gm). Further, distilled water is a more selective method of extraction than water containing electrolytes (Clemens, *et al.*, 1964).

In a series of pilot experiments where carp were forced to swim against various current velocities, the gonadal water content was proportional to the amount of exercise for a given peiod of time, as much as 5% in seven hours. A 5% change in gonadal water is about half the response we are able to induce with injections of hormones.

Ovarian Response

Ovaries of intact goldfish responded with an increase in water when injected with the same pituitary materials as the males (Text-fig. 7). The range of dosages used here tends to suggest that the response was in the "below optimum" range and that higher dosages would be necessary to produce an "optimum response." This experiment was conducted in September when the gonadal water of the female is high. As the spawning season approaches, goldfish ovarian water content decreases.

An increase in ovarian water was found to be



TEXT-FIG. 7. Gonadal hydration of female goldfish 24 hours after injections with carp pituitary extract. Standard deviations and standard errors are given.

associated with ovulation. Specially selected, "ripe" female goldfish injected with pituitary extract in February showed higher water contents than the controls; the ovulated ovaries exhibited higher water contents than those that did not ovulate[3] (Table II). The water content of the ovulated ovaries was 73.0% or above. The average ovarian water content of 35 fish that had ovulated during the course of several experiments was 75.5% (standard deviation, 2.9, standard error, .084), while 17 control fish had an ovarian water content mean of 67.9% (SD 4.365). Eight ovulating females captured in the pond in the act of spawning had an ovarian water content of 81.3% (SD 3.97, SE, .496), while nine non-spawning females taken from the pond one day later had an ovarian water content of 65.2 (SD 1.29). Thus, in both instances, ovulating females had higher ovarian water. It appears that a water increase is a part of ovulation and that this process can be stimulated by the injection of pituitary extract.

[3]When female goldfish ovulate, the eggs are released into the confines of the ovary and during the spawning act the eggs are emitted via oviducts. Since the eggs are not released into the body cavity, it was therefore possible to determine water content of the ovaries after ovulation and before spawning.

Immature Fish Response

Since the water content of the ovary decreases with the degree of ovarian development, immature females possess relatively high ovarian water. The injection of pituitary extract into immature female goldfish produced a variety of responses assumed to be related to the degree of maturity. Gonadal hydration of immature ovaries has decreased with the dosage in some experiments, increased in others, and given little or no response in still others, leaving the hypothesis of the role of hydration of immature ovaries still in a formative stage.

Likewise, the gonadal water content of immature male goldfish does not conform with that of mature specimens. Therefore, immature fish should not be used in the same experiment with adult specimens in bioassay work.

Table II. The Difference Between the Water Content of Ovulated and Non-ovulated Ovaries in Goldfish

Dosage μg/gm	% H_2O Ovaries	Ovulation	Remarks	G.S.I.
20	69.3	no		.123
20	73.9	yes	complete	.131
20	73.0	yes	partial	.158
10	75.3	yes	complete	.166
10	74.8	yes	complete	.189
10	72.6	no		.163
0	67.0	no		.168
0	67.2	no		.165
0	69.4	no		.176

Effect of Handling on Gonadal Hydration

Mature goldfish averaging 42 gm and mature carp averaging 190 gm were taken on March 6 from one of our ponds with a water temperature of 4.0° C, and allowed to acclimate to 21.0° C over a period of several hours. The fish were left one week in the environmental conditions in which the experiment was to be run.

The experiment was designed to provide controls of four types, each establishing an important reference point along the progression of experimental procedure. The dummy-injected animals were intended as the direct control on the experimental fish. These fish received completely identical "handling," including the shock of needle puncture and the carrier injections of distilled water. Carp were handled in precisely the same manner and at the same time as the goldfish, although the handled controls received no needle puncture.

The handled fish were intended as a control for all aspects of handling on the dummy-injected fish, including the shock of needle puncture but not the distilled water injection.

The "undisturbed" fish were intended as a control for handling on the handled fish. These fish were collected at the same time as the dummy-injected, handled and experimental animals. At this point, however, they were not fin-clipped, weighed or sexed, but were expeditiously placed into a separate tank beside the experimental and other control tanks with temperature, illumination and number of fish identical. On inspection day, fish were drawn with minimum handling and killed until eight males and eight females were obtained. No last minute difference in handling, such as attempts to sex these fish by hand-stripping, was introduced.

The pond fish were intended as a control for the undisturbed fish. These fish were sacrificed after being brought into the laboratory late one afternoon and kept overnight in circulating pond water.

Index cards were marked with the sex, dosage and fin-clip. The cards were mixed and used randomly to assign the treatment for each fish. When killed, the fish were taken randomly from the various tanks a few at a time so that no one individual or group had extra time in the tank. Three levels of injection were selected. The top level, 10 μg of dried pituitary powder per gm body weight, which was known to be a reliable minimum dosage, and the two levels below this (1.0 μg and 0.1 μg/gm) were questionable since they were considered to be above the response threshold of some fish and below that of others. It was thought that these dosages might produce greater variation than higher hormone levels and would be useful in variance analyses.

Carp received 0.01 cc/gm, while the goldfish received half that volume. Both were injected intraperitoneally in the axis of the pelvic fin. Twenty-four hours after injection the gonads of all fish were extirpated and a portion was used to determine loss of weight on drying, which was accepted as the relative water content.

Goldfish Females.—There appeared to be no significant difference in the ovarian water content of females receiving a distilled water injection and females receiving only a needle puncture (Table III). For this reason it appears that the carrier-injected or the handled fish could serve as controls.

An appreciation of the effect of handling is obtained from a comparison of the ovarian water content of the handled fish and the undisturbed fish (Text-fig. 8). Apparently, handling subjects the fish to sufficient stress to cause a significant reduction (2.7%) in ovarian water. The ovaries of females receiving 10μg/gm were hydrated a significant amount over those of any other group. This amounted to 2.6% over the pond females which were the next highest group, and—more significantly—7.2% over the carrier-injected controls.

The effect of the pituitary extract apparently countered the effect of the stress. The 0.1 μg/gm and 1.0 μg/gm dosages seemed to offset the effect of stress from handling, since no significant change in the ovarian water content nor significant variability from fish to fish was found between the fish injected at these levels and the undisturbed controls.

Goldfish Males.—In some respects, similar effects were observed in the male (Table III). There was no significant difference in the testicular water content between the pond group and the undisturbed group, nor between the handled

Table III. Gonadal Water Percentages of Carp and Goldfish Under Different Experimental Treatments

	Male Carp				Male Goldfish			
Treatment	% H_2O	Variance	No. Fish	"t" .05 level	% H_2O	Variance	No. Fish	"t" .05 level
Pond	74.6	2.198	11		79.0	0.737	10	
				+				—
Undisturbed	77.9	0.280	8		78.8	3.691	9	
				—				+
Handled	77.4	1.590	8		80.4	1.091	8	
				—				—
Dummy-injected	77.3	1.262	7		79.9	1.211	8	
				—				—
0.1 μg/gm	77.6	5.368	8		79.0	1.810	9	
				+				—
1.0 μg/gm	80.8	3.828	10		79.4	1.096	10	
				+				+
10 μg/gm	83.9	6.603	8		83.1	3.082	10	
	Female Carp				Female Goldfish			
Treatment	H_2O %	Variance	No. Fish	"t" .05 level	% H_2O	Variance	No. Fish	"t" .05 level
Pond	68.4	3.736	6		72.1	7.727	9	
				+				—
Undisturbed	65.5	1.308	6		70.4	12.623	8	
				—				+
Handled	66.3	1.250	8		67.7	4.404	8	
				+				—
Dummy-injected	64.9	1.905	7		67.5	1.694	8	
				—				+
0.1 μg/gm	66.3	3.096	11		69.8	9.863	10	
				+				—
1.0 μg/gm	68.3	3.683	8		68.9	13.112	10	
				+				+
10 μg/gm	70.7	7.516	7		74.7	13.747	7	

group and the dummy-injected group. Male fish showed a significant increase in gonadal water at the highest injection level, as did the females.

However, the effect of handling appears to cause a response in the male opposite to that in the female (Text-figs. 8 & 9). Gonadal water was significantly more (1.6%) in males and less (2.7%) in females after handling.

Carp Females.—The ovarian water content of the dummy-injected carp was significantly lower (1.4%) than that of carp receiving the same treatment, excluding needle puncture and distilled water (Table III).

A significant decrease (2.9%) in the ovarian water content was found in females brought from the pond into the laboratory and subjected to temperature and minimal handling (Text-fig. 10).

However, we detected no measurable differences in the ovarian water content of minimally handled fish and those experimentally handled.

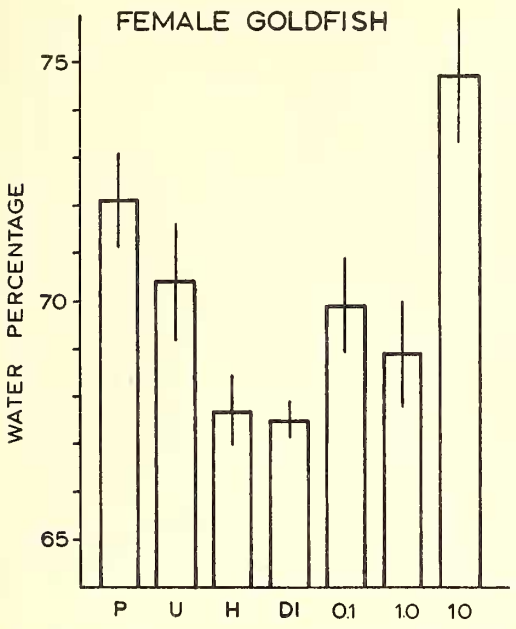
Pituitary extract injection of sufficient dosages (10 and 1.0 μg/gm) significantly raised the ovarian water content over the carrier-injected controls.

Carp Males.—The effect of bringing male carp from the pond into the laboratory and increasing the temperature increased the gonadal water content (3.3%) rather than decreasing it as in the female (2.9%) (Text-figs. 10 & 11).

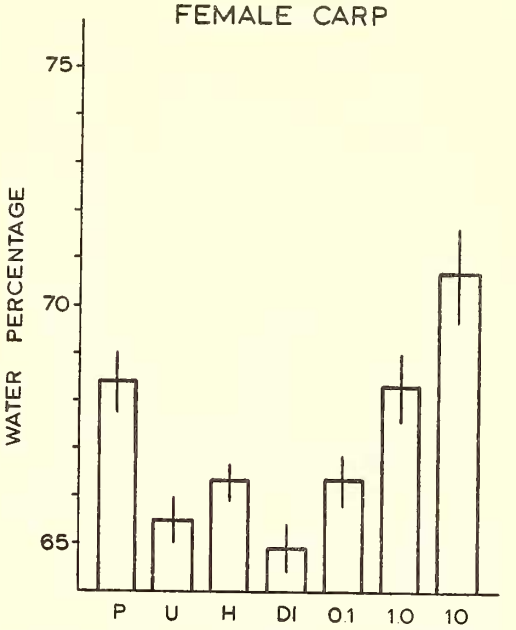
The effect of handling and dummy injecting male carp did not change the gonadal water content measurably over the undisturbed controls. However, the variances in the two former groups were significantly greater (Table III).

Males injected at the medium and higher levels exhibited significantly greater amounts of testicular water than the carrier-injected controls. The lower injection apparently had no measurable effect.

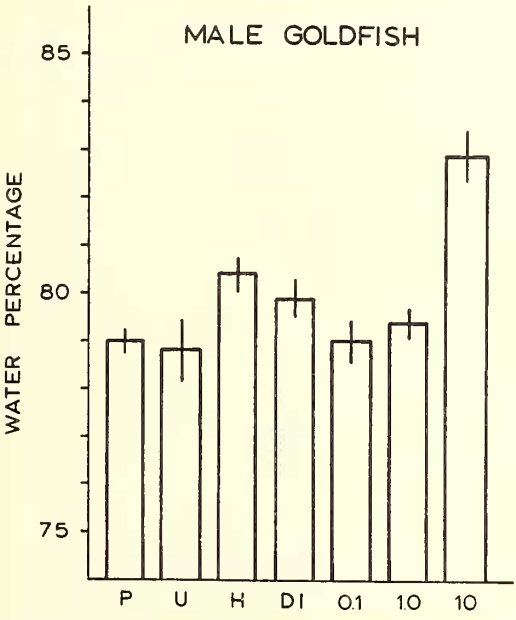
Discussion.—These experiments were designed to determine the effect of handling on the go-



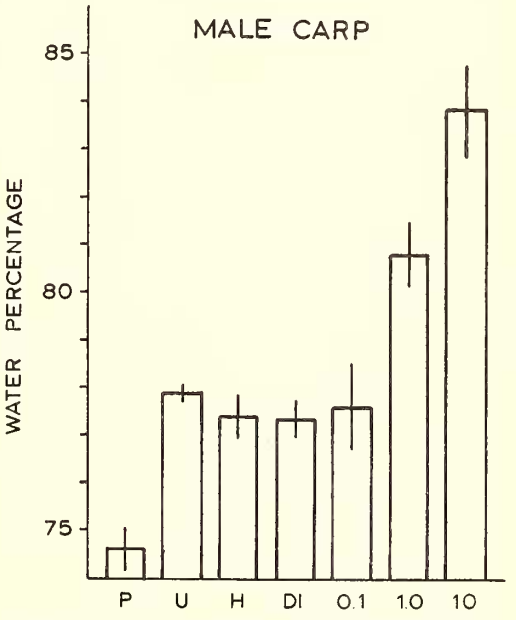
TEXT-FIG. 8. Ovarian water percentages of goldfish from the pond (**P**), "undisturbed" (**U**), experimentally handled (**H**), dummy-injected (**DI**); and injected with pituitary extracts of 0.1, 1.0, and 10 μg/gm body weight respectively. The vertical lines on the bars represent standard errors.



TEXT-FIG. 10. Ovarian water percentages of carp from the pond (**P**), "undisturbed" (**U**), experimentally handled (**H**), dummy-injected (**DI**); and injected with pituitary extracts of 0.1, 1.0, and 10 μg/gm body weight respectively. The vertical lines on the bars represent standard errors.



TEXT-FIG. 9. Testicular water percentages of goldfish from the pond (**P**), "undisturbed" (**U**), experimentally handled (**H**), dummy-injected (**DI**); and injected with pituitary extracts of 0.1, 1.0, and 10 μg/gm body weight respectively. The vertical lines on the bars represent standard errors.



TEXT-FIG. 11. Testicular water percentages of carp from the pond (**P**), "undisturbed" (**U**), experimentally handled (**H**), dummy-injected (**DI**); and injected with pituitary extracts of 0.1, 1.0, and 10 μg/gm body weight respectively. The vertical lines on the bars represent standard errors.

nadal water content of carp and goldfish. The results indicate that the effect of stress induced by experimental handling was opposite in the sexes in both carp and goldfish. The male and female response to stress is probably different only in ovarian and testicular reaction, which is governed by inherent differences in the two types of tissue; probably, stress simply augments the natural process occurring at the time. Prior to this experiment, weekly samples from the pond showed that the relative water content of the gonad was increasing in males and was decreasing in females. We believe the weekly pond samples will demonstrate that the decreasing ovarian water content at this time of the year is the change in the ratio of the cellular water to yolky material. This decrease is followed by an increase in ovarian water content with natural or artificial spawning. We believe the increased ovarian water percentage at spawning is extracellular, homologous to the seminal plasma of males.

Apparently similar handling procedures affected goldfish to a different degree than carp. Being brought into the laboratory and subjected to a temperature change, along with weighing, sexing, fin-clipping and needle puncture, caused a measurable change in the gonadal water content of goldfish. Simply bringing them into the laboratory and subjecting them to the same temperature change did not. Contrary to the observed effects in goldfish, the significant gonadal changes in carp occurred between the pond fish and the undisturbed fish rather than the undisturbed and handled or dummy-injected ones. Evidently, the effect of stress from the temperature increase did not play a major role in producing gonadal water fluctuations in goldfish.

In carp, the effects induced by a temperature increase and those induced by handling are inseparable under the conditions of the experiment, but it is believed that the stress of the laboratory rather than the stress from a temperature increase was the major factor. Certainly under laboratory conditions carp exhibit much "wilder" behavior than goldfish.

Temperature changes, however, should not be eliminated entirely as a stress factor since Hoar & Cottle (1952) reported that the water content of muscle and liver of goldfish varied directly with the change in acclimatization.

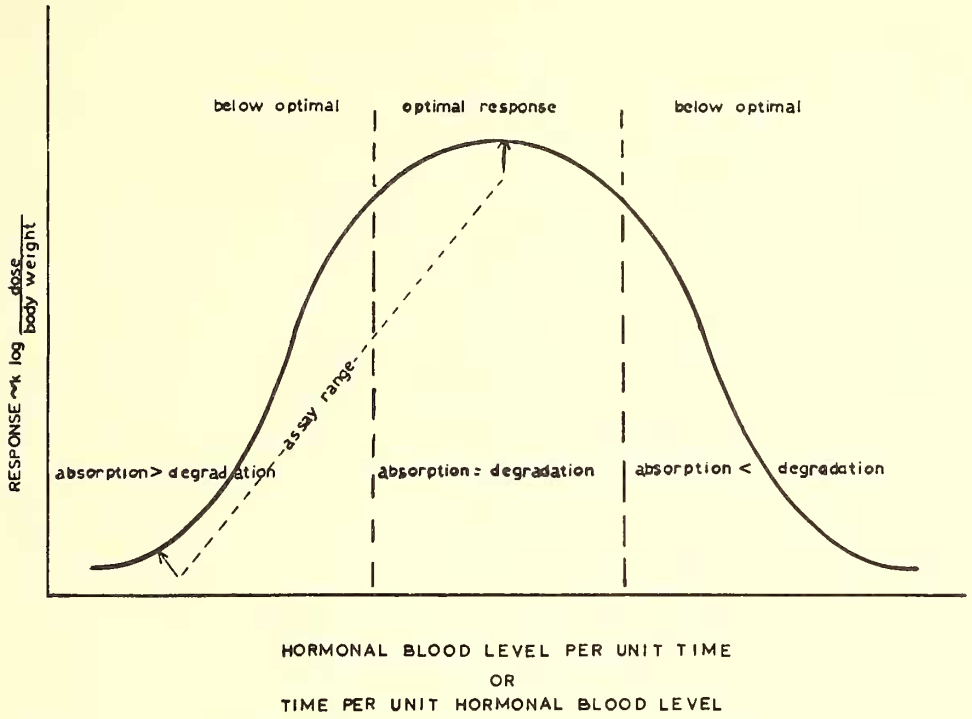
There is no apparent reason why carrier-injected female carp had a significantly ($p=.025$) lower ovarian water content than the handled controls, although male carp and both sexes of goldfish showed no measurable change between the gonads of the two control groups. This suggested that female carp may be more susceptible to stress than males.

There is good evidence that in fishes as in higher vertebrates, adrenocortical hormones are involved in stress, the regulation of metabolism, and the hydromineral balance (see reviews by Pickford & Atz, 1957; Fontaine, 1956; Chester Jones, Phillips & Holmes, 1959; Ball, 1960; and Chester Jones & Phillips, 1960). Both glucocorticoids and mineralocorticoids are known in fishes. Cortisol and cortisone have been found in the blood of carp; although Chester Jones & Phillips suggest it to be universally present in fish, to date aldosterone has been identified only in salmon plasma. Hatey (1958), as reported by Chester Jones & Phillips (1960), was able to detect different levels of 17-hydroxycorticosteroid in the plasma of "unstressed" carp, slightly stressed carp and carp stressed by forced swimming. That such a system could be operating in the regulation of gonadal water awaits future experimentation, and the establishment of the physiological roles of the corticosteroids in fish.

Discussion

The purpose of this study was to pursue the role of the gonadotropins in the reproductive cycle of fish, particularly with respect to water (and electrolyte) movement in the gonads. The data collected established that the testes undergo changes in water content in a way similar to the changes observed in the seminal plasma (Clemens & Grant, 1964). We suspect the same processes are involved in both changes and the observations on the gonads merely reflect the changes of the seminal plasma. The anatomy of the gonads is consistent with this view, since the semen is stored in the seminiferous tubules which are an integral part of the testes. In the female, an ovarian increase in water at the time of ovulation has been established. In both male and female goldfish the gonadal water changes were observed in nature and can be induced by the injection of water extracts of whole ground acetone-dried pituitary of carp. We are confident, then, that the observed responses, gonadal hydration changes in both sexes, play an important role in fish reproduction—in the male it is the preparation of the semen for discharge and in the female it is ovulation.

Since bioassays usually involve placing a given quantity of hormone in an animal, we can assume that as the hormone is absorbed there is a rise in the blood level to a maximum with a subsequent fall (Text-fig. 12). We can relate the rise in the level to hormone absorption and the fall in the level to hormone degradation and refer to three distinct stages, (1) absorption rate greater than degradation rate, (2) absorption equal to degradation, and (3) absorption less than degrad-

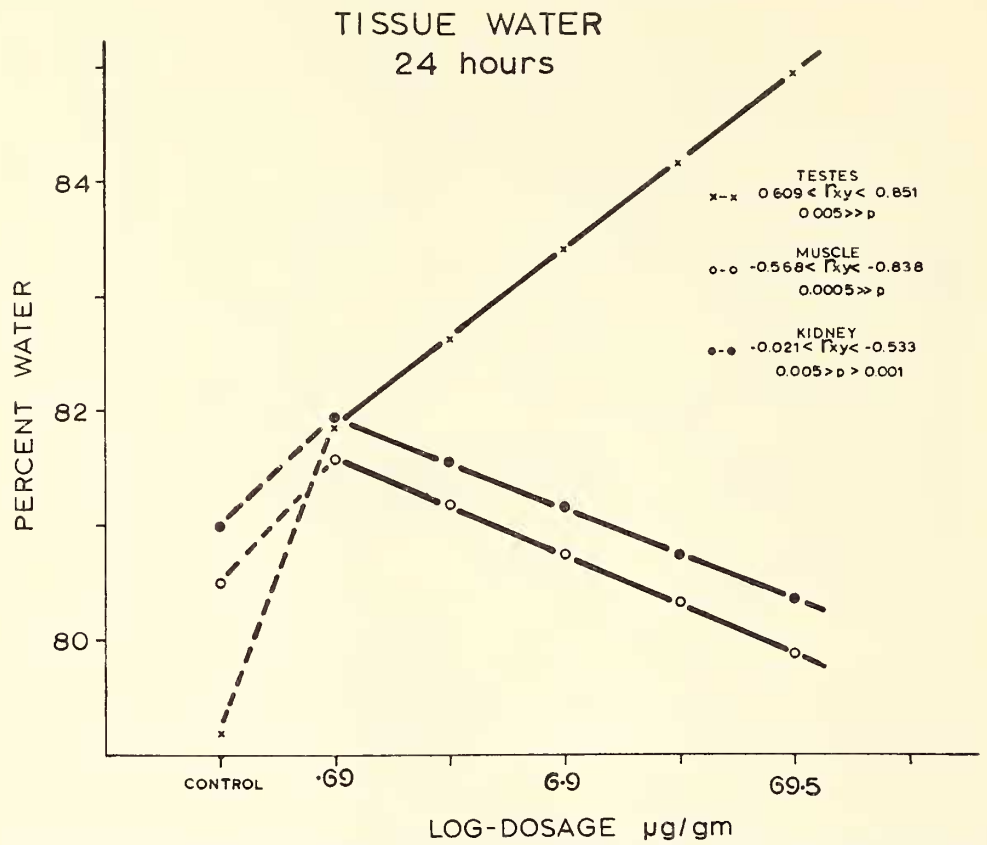


TEXT-FIG. 12. Theoretical curve after hormonal injection.

ation. We think the seminal fluidity response with respect to time (Text-fig. 1) reflects in general the blood level of the hormone. We realize that the graphic figuration of the hormone level may not coincide with that of the response level but until this relationship is learned, one might be used as the approximation of the other.

The role of the endocrines in water and electrolyte metabolism in fish has received considerable attention and in the reviews of Fontaine (1956), Pickford & Atz (1957), Black (1957), and Chester Jones, Phillips & Bellamy (1962), to mention a few, gonadal hydration is an unknown subject. Likewise, in the reviews of fish gonadotropins (Pickford & Atz, 1957; Hoar, 1957; Pickford, 1959; Ball, 1960; and Marshall, 1960), only vague or incidental references are made to this subject. Other organs have received attention. For this reason, the water content of the kidney, muscle and brain of carp (same fish as reported pages 194 and 196) were studied with respect to gonadal hydration. Changes occurred in these organs, with the exception of the brain in which there apparently is no response. The changes that occurred in the muscle and the kidney varied with the size of the injection. Twenty-four hours after injection, the water of the muscle and the kidney showed a decrease with increasing injection (Text-fig. 13), a response opposite to the gonads. The eight-hour observations showed that the kidney and the muscle were dehydrating along with the gonad (Text-fig. 14). Thus, hydration responses in comparison to the controls were much greater for the gonad than those of the kidney and the muscle. The mechanism for hydration was observed to involve the gonad in a much wider range of hormonal levels than the muscle and kidney. Two of several lines of apparent interpretations might be mentioned here. Muscle changes appear to be a transient, passive response, a temporary shift in the water compartments resulting from the interplay of the kidney action and the circulatory system, or even from osmotic changes in the blood alone. Although this shift in water compartments involves the gonads and other tissues, it appears selective for the gonads, possibly because the gonads serve as an open-end compartment allowing a considerable differential between the inward and outward (testes to blood) movement of water. The second possibility is simply that hormonal impurities in the injection caused side effects or that other endocrine systems were brought into play as a result of the testicular response (steroids, etc.).

In experiments with goldfish, other organs, in

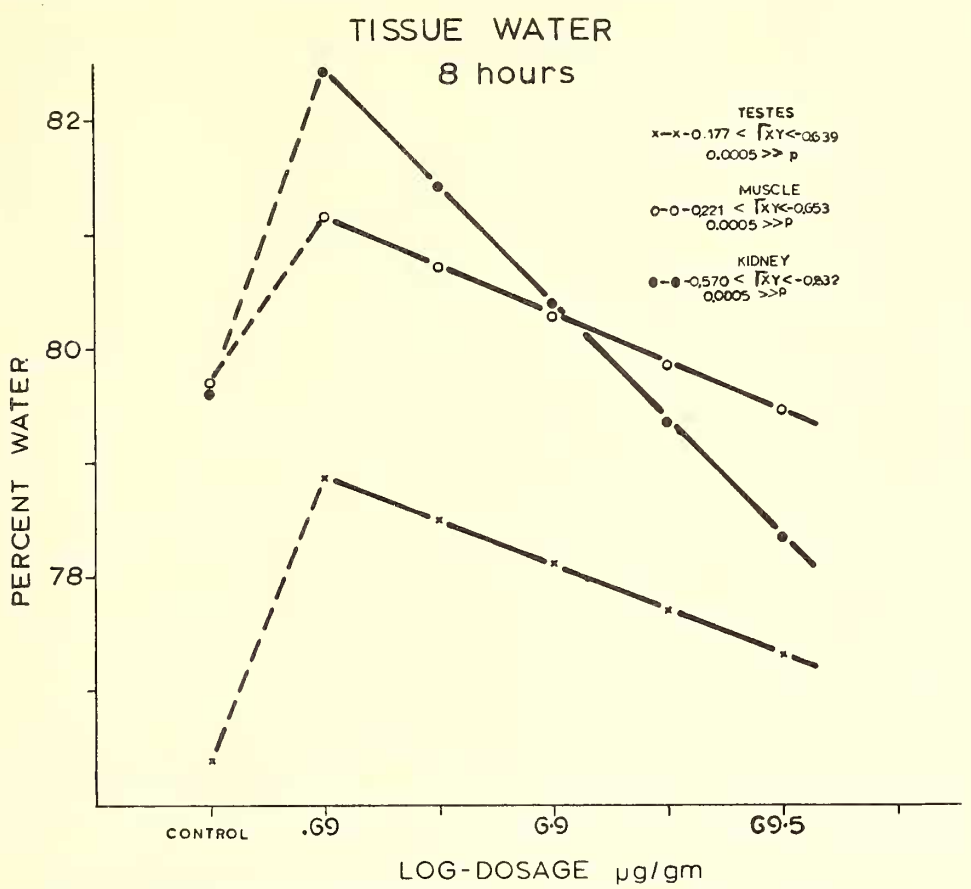


TEXT-FIG 13. Hydration of testes muscle and kidney of carp 24 hours after homoplastic injections of pituitary extract.

addition to the kidney and muscle, were shown to undergo hydration changes in proportion to the injected dosage. The opercular bone either with or without skin (Text-fig. 15) produced a more marked change than either the muscle or the kidney, and the changes corresponded closely to those observed in the testes. The blood of male carp changed with injections of pituitary extract, since the red blood count, the haematocrit and the haemoglobin were observed to rise with concomitant rises in gonadal water (Table IV).

These data on hydration changes in other parts of the body are presented as concomitant events and in a problem as complex as this they are presented only to show that gonadal hydration appears to be rather large in relation to responses in other organs. This is taken to mean that a major response is in the gonads. If the response of the gonads is one associated with spawning, as it is believed to be, then the fish at the peak of the response are expected to be in a highly active physiological state, the presumptive processes involving a number of endocrines, water and electrolyte movement. It is assumed that we are observing this in general response in the body and the gonadotropin(s) involved seems certain to affect the intake of water and electrolytes into the gonad and may do the same on a smaller scale for other body organs and/or may excite other endocrines we believe to be associated with water and electrolyte metabolism. At any rate, we believe that in a problem this complex some common denominator measuring the activity of the various endocrines at the time of spawning is needed and that water and electrolytes may serve this need. Another need is the culture of gonadal tissue with the production of normal cells so that the responses of various hormones can be observed under isolated conditions.

Mammalian vasopressin, oxytocin, pituitrin, LH, FSH, ACTH, STH, TSH and prolactin, though they were administered through a wide range of dosages, showed much smaller and less consistent changes in the testicular water that were unlike the 24-hour observations resulting from even small injections of carp pituitary. This means that goldfish were refractory to mammalian hormones at 24 hours, but these hormones



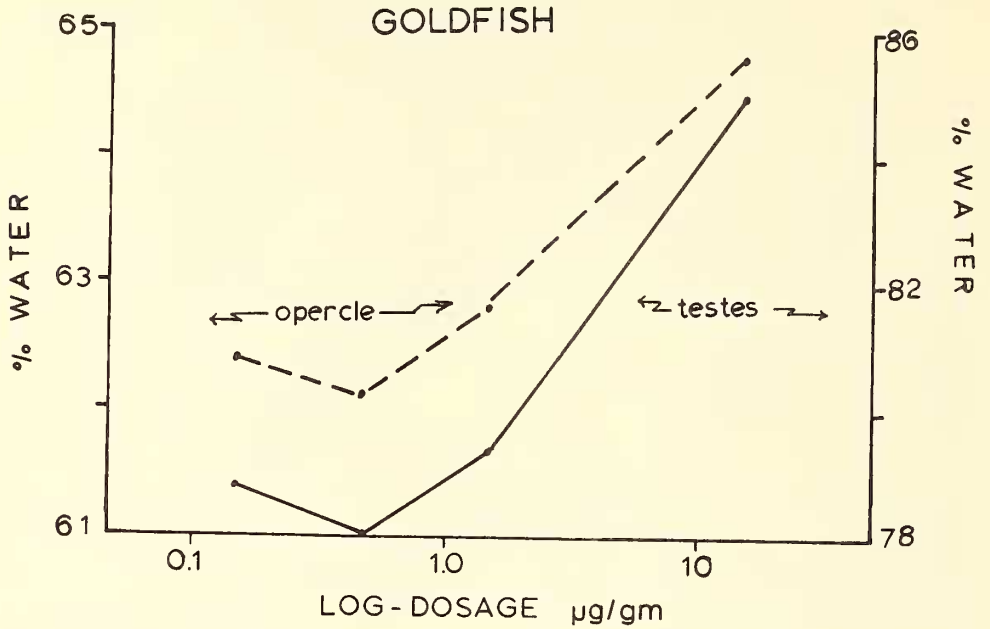
TEXT-FIG. 14. Hydration of testes, muscle and kidney of carp 8 hours after homoplastic injections of pituitary extract.

might have an effect on gonadal hydration at some other period of observation.

The reported chemical properties (Clemens, *et al.*, 1963) suggest the active principle is more like the gonadotropins than any of the other mammalian pituitary hormones. The gonadal response pattern is the basis for purification studies of the active principle now in progress in this laboratory.

The literature contains a number of instances where gonadotropins are suggested or reported as having a role in water and electrolyte metabolism. Pickford & Atz (1957) point out that a number of papers suggest (1) the pituitary of fishes is in some way concerned with calcium metabolism and that the gonadotropins may have an effect on the salt content of the blood of fishes, (2) the ability of fish to withstand or even seek changes in the salinity or osmotic pressure of the water surrounding them needs to be considered in the light of the drastically different environments encountered by anadromous and catadromous fishes on their spawning migrations. In birds, Riddle & Dotti (1945) showed that the pituitary hormone which increases plasma calcium in doves and pigeons is a gonadotropin. Breneman & Zeller (1961) and others show that P^{32} uptake in the testes of the chick is increased after injections of LH and FSH secured from the National Institute of Health. Radioactive phosphorus is taken up in the testes of goldfish in proportion to the dosage of carp pituitary extract. Rugh (1939) stimulated spermiation with hypophysial implantations and described the process in male toads. No study has been found that deals directly with gonadal hydration in fish, although there have been some indications of increased flow of semen after injections of pituitary materials (Clemens & Grant, 1964). Clemens & Sneed (1962) used an increase in seminal fluidity to indicate the presence or absence of the principle in fish pituitaries.

Our data show that hydration is an essential part of seminal plasma elaboration in the male



TEXT-FIG. 15. Opercular bone (with skin) and testicular hydration of goldfish 24 hours after injections with carp pituitary extract.

and ovulation in the female and is hormonally controlled. The hormone regulating the hydration process appears to be a gonadotropin and it is a matter of fact to suspect the involvement of electrolytes and other endocrines.

ACKNOWLEDGMENTS

We acknowledge our appreciation to Dr. Arthur Ghent for his statistical advice, Dr. Max Wilcomb for his participation in the P^{32} experiments, Messr. David Adamson and Lloyd Berry for their assistance in collecting the blood data, Mr. Daniel Zellmer for taking part as a National Science Foundation Undergraduate Research Participant in the forced exercise studies, and to Mr. Waynon Johnson who contributed a part of the data incorporated into the standard curve and collected while working on another phase of our gonadotropin studies.

The original stock of carp for our ponds was provided by Mr. O'Reilly Sandoz, Oklahoma Department of Wildlife Conservation, and Mr. James Savage, Resident Engineer, Altus-Lugert Project, U. S. Corps of Army Engineers.

To the many other persons who assisted in a variety of ways, our heartfelt thanks.

SUMMARY

1. The seminal plasma elaboration in prespawning carp and goldfish is reflected by a gonadal hydration, since the seminiferous tubules in these species are an integral part of the testes.

2. With injections of carp pituitary extract, the gonadal hydration undergoes a gradual rise and fall through a period of three days and is believed to approximate the level of the hormone

TABLE IV. A COMPARISON OF THE CHANGES IN THE BLOOD AND GONADS OF MALE CARP AFTER INJECTION OF HOMOPLASTIC PITUITARY EXTRACT

Dosage µg/gm	% H_2O Testes	% H_2O Blood	Hematocrit % cells	Hemoglobin gm/100 ml	Red Blood count millions/mm^3	Fish No.
0	76.1	85.3	35.0	8.33	1.52	3
1	78.2	82.5	34.3	8.08	1.84	3
10	83.3	79.5	38.3	8.95	1.96	3
100	84.9	84.0	39.2	9.88	2.03	3
316	85.3	85.1	36.8	8.88	2.08	3

in the blood. Twenty-four-hour responses provide an ideal bioassay for following the hormones in chemical isolation procedures.

3. Responses can be obtained throughout the year, although the periods of greatest response appeared during May, June and October.

4. If gonadal hydration is hormonally regulated, then its study in control fish and in pondfish (fish in nature) should reflect the endogenous hormone level.

5. The control fish showed a greater seasonal variation than the injected fish and a reduction of gonadal water below the pondfish during the period from April through June which appeared to be traced to insufficient food, although the feeding rate was the same throughout the year. Goldfish apparently need more food during the prespawning period. Gonadal water of starved fish decreased from 81.9 to 72.9% in 23 days.

6. The effects of bringing male goldfish from the pond into the laboratory and raising the water temperature alone caused a slight decrease in gonadal water which was not statistically significant, while the effects of bringing the fish into the laboratory and raising the water temperature along with the handling involved in their weighing, sexing, fin-clipping and needle puncture caused a 1.6% rise in the gonadal water content ($p=.05$).

7. Gonadal hydration of male goldfish in the pond was relatively high during the prespawning and spawning periods, and during the late summer and early fall.

8. It appeared that endogenous blood levels could not be altered significantly during March by raising the temperature from 3° C to 22° C, although marked changes in gonadal hydration were induced with injections at temperatures of 16.0 and 22.0° C and little or no response was observed at 3.0 and 10.0° C. These observations suggest that temperature increases in nature may not excite hormonal release but merely enhance the effect of the release.

9. Gonadal fat was carefully dissected away from any portion of the gonad used in water determinations, since fat could substantially reduce the water content.

10. The gonadal water of female goldfish increases when injected with the same pituitary materials as the males. It appears that the increase of water is associated with ovulation, since the average ovarian content of females that had been induced to ovulate with injections of pituitary materials was 75.5%, and ovulating females captured in the pond in the act of spawning was 81.3% while non-spawning females had an ovarian water content of 65.2%.

11. The effect of stress induced by experimental handling was opposite in the sexes in both carp and goldfish. In females a small but significant decrease in ovarian water was observed while in males the observed response was a small but significant increase in testicular water.

12. Apparently similar handling procedures affected goldfish to a different degree than carp. Being brought into the laboratory and subjected to a temperature change, along with weighing, sexing, fin-clipping and needle puncture, caused a measurable change in the water content of goldfish. Simply bringing them into the laboratory and subjecting them to the same temperature change did not. Contrary to the observed effects in goldfish, the significant gonadal changes in carp occurred between the pond fish and the undisturbed fish rather than the undisturbed and the handled or dummy-injected ones. Evidently temperature did not play a major role in producing gonadal water fluctuations in goldfish but the effects of temperature increase and those of handling were inseparable in carp under the conditions of the experiment.

13. Hydration changes concomitant with those of the gonads were observed in the muscle, kidney, bone and blood but were relatively small in relation to those of the gonad.

14. The hormone regulating the hydration process appears to be a gonadotropin and it is a matter of fact to suspect the involvement of electrolytes and other endocrines.

References

Ball, J. N.
1960. Reproduction in female bony fishes. Symp. Zool. Soc. London, 1: 105-135.

Black, V. S.
1957. Excretion and osmoregulation. *In*: "The Physiology of Fishes, I," 163-205. Academic Press, New York. pp. 447.

Breneman, W. R., & Zeller, F. J.
1961. P^{32} uptake by chick testes as an endpoint for gonadotropin assay. American Zool.: 346 (abstract).

Chester Jones, I. & Phillips, J. G.
1960. Adrenocorticosteroids in fish. *In*: "Hormones in Fish." Symp. Zool. Soc. London, 1: 17-32.

Chester Jones, I., Phillips, J. G. & Bellamy, D.
1962. Hormones affecting environmental adjustment—Studies on water and electrolytes

in cyclostomes and teleosts with special reference to *Myxine glutinosa* L. (the hagfish) and *Anguilla anguilla* L. (the Atlantic eel). Gen. Comp. Endocrinol. (Suppl.), 1: 36-47.

Chester Jones, I., Phillips, J. G. & Holmes, W. N.

1959. Comparative physiology of the adrenal cortex. *In*: "Symposium of Comparative Endocrinology." John Wiley & Sons, New York. pp. 746.

Clemens, H. P., Ciereszko, L. S., Shoemaker, J. & Grant, F. B.

1964. Partial characterization of the gonadal hydration principle in pituitaries of carp, (*Cyprinus carpio*). Gen. Comp. Endocrinol. 4: 503-507.

Clemens, H. P. & Grant, F. B.

1963. The seminal fluidity response of carp (*Cyprinus carpio*) and rainbow trout (*Salmo gairdneri*) as a biological assay for a pituitary hormone. (In press: Copeia).

Clemens, H. P. & Sneed, K.

1964. The seminal fluidity response of carp to Spawn Warm-Water fishes. U. S. Dept. Interior, Fish and Wildlife Service, Research Report 61. pp. 30.

Fontaine, M.

1956. The hormonal control of water and salt electrolyte metabolism in fish. Mem. Soc. Endocrinol., 5: 69-82.

Hoar, W. S.

1957. Endocrine organs. *In*: "The Physiology of Fishes, I," 245-285. Academic Press, New York. pp. 447.

Hoar, W. S. & Cottle, M. K.

1952. Some effects of temperature acclimatization on the chemical constitution of goldfish tissues. Canadian J. Zool., 30: 49-54.

Leloup-Hatey, J.

1958. Influence de l'agitation motrice sur la teneur du plasma en 17-hydroxycorticosteroids d'un Téléostéen: la Carpe (*Cyprinus carpio* L.). C. R. Acad. Sci., Paris. 246: 1088.

Marshall, A. J.

1960. Reproduction in male bony fish. Symp. Zool. Soc. London, 1: 137-151.

Pickford, G. E.

1959. The nature and physiology of the pituitary hormones of fishes. *In*: "Symposium of Comparative Endocrinology." 404-420. John Wiley & Sons, New York. pp. 746.

Pickford, G. E. & Atz, J. W.

1957. "The Physiology of the Pituitary Gland of Fishes." New York Zool. Soc., New York. pp. 613.

Riddle, O. & Dotti, L.

1945. Pituitary and sex hormones which increase plasma calcium in birds and mammals. Proc. Amer. Phil. Soc. 89: 499-516.

Rugh, R.

1939. The reproductive processes of the male frog, *Rana pipiens*. J. Exp. Zool. 80: 81-105.